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A Short-activating RNA Oligonucleotide Targeting the Islet β -cell Transcriptional Factor MafA in CD34⁺ Cells

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Upon functional loss of insulin producing islet β -cells, some patients with diabetes become dependent on life-long insulin supplementation therapy. Bioengineering surrogate insulin producing cells is an alternative replacement strategy. We have developed a novel approach using short-activating RNA oligonucleotides to differentiate adult human CD34⁺ cells into insulin-secreting cells. By transfecting RNA to increase transcript levels of the master regulator of insulin biosynthesis, v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), several pancreatic endodermal genes were upregulated during the differentiation procedure. These included Pancreatic and duodenal homeobox gene-1 (PDX1), Neurogenin 3, NeuroD, and NK6 homeobox 1 (NKX6-1). Differentiated CD34⁺ cells also expressed glucokinase, glucagon-like peptide 1 receptor (GLP1R), sulfonylurea receptor-1 (SUR1) and phogrin—all essential for glucose sensitivity and insulin secretion. The differentiated cells appropriately processed C-peptide and insulin in response to increasing glucose stimulation as shown by enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting analysis, western blotting, and immunofluorescence staining. We provide a new approach using short-activating RNA in developing insulin producing surrogate cells for treating diabetes.

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Subject Category: siRNAs, shRNAs, and miRNAs; Therapeutic Proof-of-concept

Introduction

Insulin is produced by the β -cells of the pancreas and regulates cellular uptake of glucose from the blood. Type-1 diabetes mellitus (T1DM) is the result of autoimmune destruction of pancreatic β -cells resulting in insulin deficiency and without prompt treatment patients may develop life threatening diabetic ketoacidosis. In these patients, it is imperative to exert tight glycemic control in order to prevent numerous macrovascular and microvascular complications including cardiovascular disease, renal disease, neuropathy or retinopathy. Progress in the pharmacology of insulin replacement has led to the development of new insulin formulations with improved pharmacokinetic and pharmacodynamic properties. However, despite this, patients with T1DM who require painful daily injections frequently expose themselves to hypoglycaemic episodes.¹ In addition, adequate control of blood sugar levels is often difficult because synthetic insulin injections are not able to replicate the glucose regulatory function of normal islet cells.² Currently, transplantation of cadaveric pancreatic islets is the preferred cell replacement therapy for T1DM.³ However, this approach is limited by the need for immune suppression and a shortage of donor tissue suitable for transplantation. Other sources of surrogate cells that have been considered include pluripotent embryonic stem cells,^{4,5} *ex vivo* expansion of β -cells,⁶ use of endocrine progenitor cells,⁷ transdifferentiation of liver and intestinal cells^{8,9} or bone marrow mesenchymal stem cells.¹⁰ It is, however, now recognized that embryonic or bone marrow derived stem cells harbor the

greatest potential for cell replacement strategy as they can be expanded to therapeutically relevant numbers and has the plasticity to generate most cell types of the body.

The process by which the pancreas arises from the definitive endoderm derived primitive gut tube is extensively defined in the literature. During development of the pancreas, the dorsal and ventral protrusion of the primitive gut epithelium gives rise to the endodermal germ layer where sonic hedgehog signaling is suppressed by activin A and basic fibroblast growth factor (bFGF) to allow development of the definitive endoderm.¹¹ Noggin, epidermal growth factor (EGF), bFGF further directs differentiation towards pancreatic and duodenal homeobox gene-1 (PDX1) expressing posterior foregut endodermal cells.¹² PDX1 controls the balance between exocrine and endocrine cells where it allows the expansion of an undifferentiated pancreatic progenitor population which can either be directed towards α -pancreatic or δ -pancreatic cells.^{13–15} Neurogenin 3 and NeuroD initiates progression towards β -cells from the pancreatic endoderm. NKX6-1 coordinates β -cell specific islet development together with v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) (a β -cell specific transcription factor that binds to RIPE3b, a conserved enhancer element that regulates expression of insulin) for functional maturation and acquisition of glucose sensitivity.^{16,17}

By using a sequential cocktail of growth factors and bioactive small molecules, D'Amour *et al.*, successfully drove human embryonic stem cells through key developmental steps of the pancreas, including the definitive endodermal

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stage through to the foregut; pancreatic endodermal; endocrine progenitor stage; and finally to pancreatic β -cells.^{12,18–20} Despite creating differentiated cells expressing insulin levels similar to that of human islet cells, they only released biologically active insulin in response to various secretagogues but not in response to glucose. As the major limitations of using embryonic stem cells in clinical therapy include their controversial source and tumorigenic potential,^{13,21} their usefulness for regenerative therapy in the context of chronic non-malignant disease is still contentious. Human adult bone marrow derived stem cells, in contrast with their recently identified pluripotency²² and proven safety profile, are an attractive alternative to embryonic stem cells. Adult human stem cells isolated from the bone marrow can, under appropriate conditions, differentiate into multiple cell types including cartilage, bone and adipose tissue.¹³ Chandra *et al.*,²³ recently demonstrated that human adipose tissue derived stem cells exposed intermittently between serum rich and serum-free medium in the presence of differentiation factors, were successfully differentiated into functional islet like aggregates that synthesized C-peptide and insulin in a glucose dependent manner. Like the other studies mentioned, certain non-GMP (good manufacturing practice) compliant factors including β -mercaptoethanol used in the differentiation protocol currently limits clinical applicability.²³ More recently, targeted activation of crucial pancreatic transcription factors successfully generated insulin-secreting cells;^{24–27} however, because these methods depend largely on the use of lentiviral vectors, their potential for clinical use is restrained.^{28–31}

In human cells, it has been reported that short RNA to the promoter regions of certain genes can activate expression at the transcriptional level without altering the genome.^{32,33} This process has been referred to RNA activation (RNAa), and is shown to be conserved in other mammals including mice, rats and primates.³⁴ Although the exact mechanism of action is still not fully defined, it is suggested that naturally occurring antisense transcripts from the same gene loci or within close vicinity are able to recruit argonaute proteins and histones methyltransferases (proteins responsible for epigenetic gene silencing). Short-activating RNAs (saRNAs) therefore may positively regulate transcription by targeting these antisense molecules for degradation, resulting in upregulation of sense messenger RNA, thus reversing gene silencing.^{35–37} On the basis of this principle, we have developed a method of designing saRNAs for positively regulating specific genes with the aim of reprogramming human adult stem cells. For this study, we have used a subpopulation of adherent CD34⁺ cells mobilized from normal adult human bone marrow as they are known to have multi-lineage differentiation potential. These adherent CD34⁺ cells have previously been characterized as having greater plasticity than the non-adherent population as they express determinants specific for differentiation towards liver, pancreas, heart, muscle, and nerve cells.^{38,39} We have successfully induced the differentiation of adherent CD34⁺ cells towards an endodermal pre-proinsulin expressing lineage using a serum-free culture system supplemented with a combination of GMP compliant differentiation factors traditionally used for culturing pancreatic endocrine cells (activin A, exendin-4, noggin, EGF, and bFGF). In addition, as it has recently been reported that saRNA oligonucleotides

can mediate induction of endogenous pluripotency factors in human mesenchymal stem cells,⁴⁰ the data presented here show for the first time that saRNA oligonucleotides designed to upregulate mRNA transcripts of the islet β -cell specific transcription factor, MafA profoundly influences the differentiation of human adult CD34⁺ cells.

Results

Differentiation and characterization of CD34⁺ cells using defined factors and MafA saRNA

To recapitulate the series of gene activation that occurs during commitment of progenitor cells toward the pancreatic endodermal lineage, we used freshly isolated adherent adult human CD34⁺ cells as they display better multi-lineage potential.³⁹ Adherent CD34⁺ cells were exposed to differentiation factors defined for inducing insulin-expressing cells. This included activin A, exendin-4, noggin, EGF, and bFGF (**Figure 1a**). As cells grown under these conditions have previously been induced to express insulin, we also confirm that adherent CD34⁺ cells showed positive expression of PDX1, insulin, and Rfx6 (**Figure 1b**). These cells however did not respond to glucose stimulation (data not shown). To further enhance expression of insulin and its specific transcription factors, the differentiated cells were cultured in a GMP (good manufacturing practice) grade serum-free medium and transfected at days 2, 5, and 8 with short-activating RNAs (saRNAs) designed specifically to target the islet β -cell master regulator MafA (**Figure 1a**). Here, we established that the optimal time point to establish glucose responsiveness was at day 12 after which the stability of these cells *in vitro* was severely compromised (**Supplementary Figure S1a**). Total RNA from the cultured cells was isolated at days 0, 3, 6, 9, and 12 for reverse transcription and analysis of messenger RNA levels. The primer pairs for insulin were selected to amplify exons 2 and 3 of the human insulin gene. The products therefore revealed two bands at 343 bp and 307 bp (**Figure 1b,c**). The 343 bp fragment corresponds to the human insulin mRNA spliced at position +3397 to generate full pre-proinsulin mRNA. The 307 fragment is the product of an alternative splicing pathway characterized at splice junctions +3433 and +3522 of the human insulin gene.^{41,42} The human pancreatic islet cells express insulin mRNA in its shorter form when compared with its expression in non-pancreatic cells due to the specificity of the splicing machinery in different cell types. Both 341 bp and 307 bp variants however go through complete conversion into insulin. A semi-quantitative profile of the saRNA-transfected cells showed significant upregulation of the key insulin specific transcription factors PDX1, Rfx6, MafA, and insulin by day 3 (D3) in MafA-transfected cells where this was maintained for 12 days (D12) (**Figure 1c,d–g**). Expression of these genes in MafA-transfected cells were sustained until day 12 (D12) compared with D9 in untransfected cells (**Figure 1d–g**).

Expression level of pancreatic markers in MafA-transfected cells

To consolidate this observation, a quantitative analysis of the genes was performed using untransfected and MafA-transfected cells at D12 and compared with undifferentiated CD34⁺ cells. MafA-transfected cells contained a significant

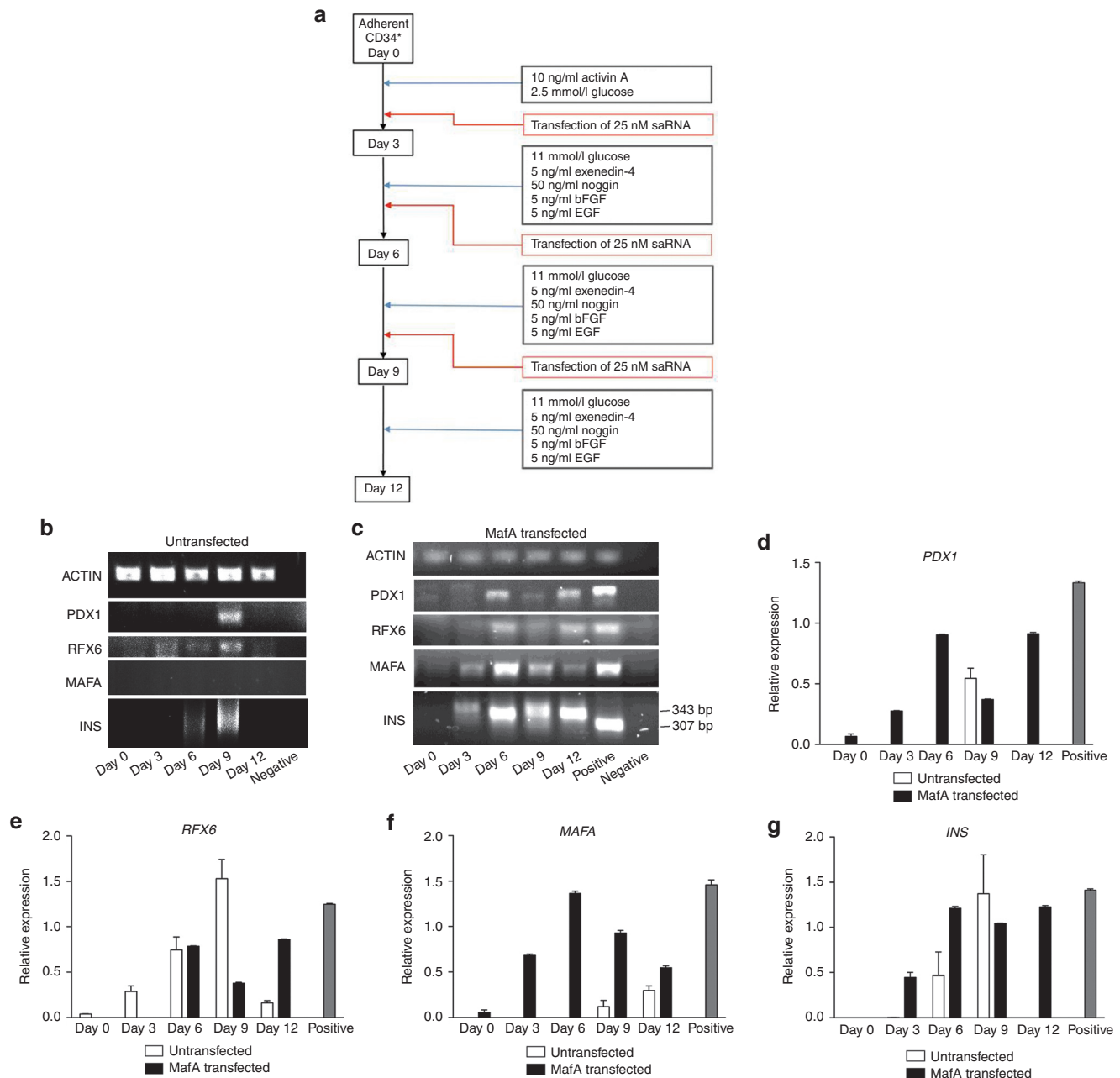


Figure 1 Differentiation procedure of CD34⁺ cells in the presence of MafA saRNA. (a) A flow diagram of the differentiation protocol including addition of the growth hormones and transfection of the short-activating RNA (saRNA) oligonucleotide specific to MafA. (b) Semi-quantitative PCR analysis of reverse-transcribed mRNA isolated from cultured CD34⁺ cells at days 0, 3, 6, 9, and 12, and (c) analysis of reverse-transcribed mRNA isolated from MafA-transfected CD34⁺ cells. Positive PCR control used was total human adult pancreatic RNA. (d–g) Semi-quantitative analysis of mRNA expression levels of (d) *PDX1*, (e) *RFX6*, (f) *MafA*, and (g) *INS*, respectively from samples as mentioned in b and c. Values are mean \pm SEM of five independent differentiation experiments. A paired *t*-test at 95% confidence gave a significant difference in expression values between untransfected and MafA-transfected cells for *RFX6* at D12 ($P = 0.03$) and at Day 9 ($P = 0.001$) and D12 ($P = 0.007$) and for *MafA*. MafA, v-maf musculoaponeurotic fibrosarcoma oncogene homologue; *PDX1*, Pancreatic and Duodenal homeobox 1; *RFX6*, Regulatory Factor x box 6.

increase in the expression of *PDX1* (Figure 2a), Neurogenin 3 (*NEUROG3*) (Figure 2b), NeuroD (*NEUROD1*) (Figure 2c), *NKX6-1* (Figure 2d), *MafA* (Figure 2e), glucokinase (*GCK*) a hexokinase enzyme which phosphorylates glucose in the initial steps of glucose metabolism⁴³ (Figure 2f), glucagon-like peptide 1 receptor (*GLP1R*), an important factor for insulin secretion (Figure 2g),⁴⁴ sulfonylurea receptor-1

(*SUR1*), *ABCC8*, a potassium channel (K-ATP) regulator of insulin secretion (Figure 2h),^{45,46} Phogrin (*PTPRN2*) an insulin granule membrane protein (Figure 2i),⁴⁷ and *INS* (Figure 2j). To confirm that the target gene effect caused by the saRNA was not only specific to CD34⁺ cells, we demonstrated that transfection of the oligonucleotide into a hepatocyte cell line (HepG2) also induced a positive regulation

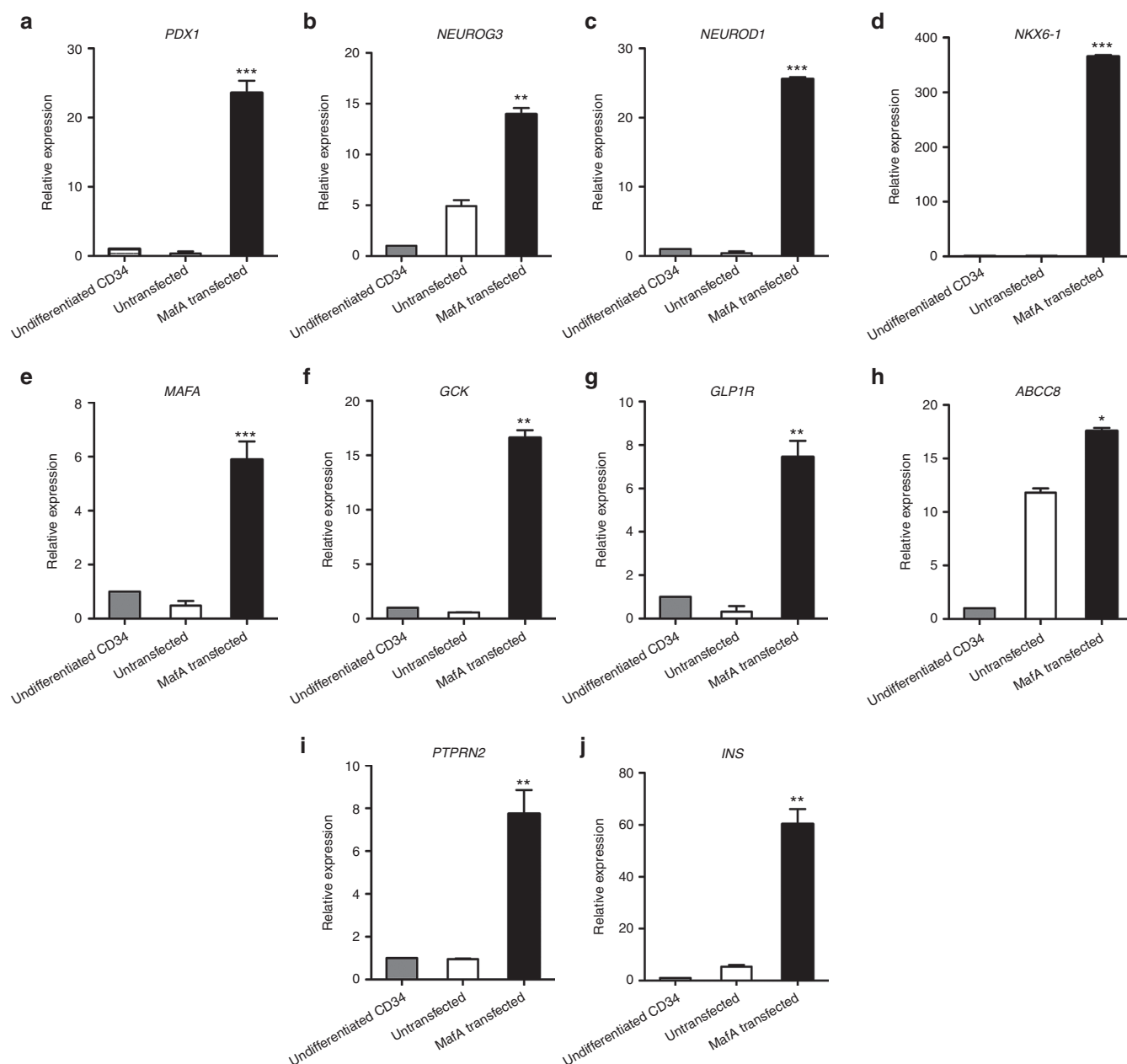


Figure 2 Upregulation of key transcriptional factors in the presence of MafA saRNA. mRNA levels of (a) *PDX1*, (b) *NEUROG3*, (c) *NEUROD1*, (d) *NKX6-1*, (e) *MafA*, (f) *GCK*, (g) *GLP1R*, (h) *SUR1* (K-ATP component (sulfonyl urea receptor, the ATP sensitive potassium channel), *ABCC8*, (i) *PTPRN2*, and (j) *INS*. Values are mean \pm SEM of three independent experiments run in triplicate and shows expression relative to undifferentiated CD34⁺ cells. * $P < 0.005$; ** $P < 0.0001$; *** $P < 0.00001$, paired Student's *t*-test, two-tailed at 95% CI. *GCK*, glucokinase; *GLP1R*, glucagon-like peptide 1 receptor; *INS*, insulin; MafA, v-maf musculoaponeurotic fibrosarcoma oncogene homologue; *NEUROD1*, NeuroD; *NEUROG3*, Neurogenin 3; *PDX1*, Pancreatic and Duodenal homeobox 1; *PTPRN2*, Phogrin; *SUR1*, sulfonylurea receptor-1.

of *PDX1* and Neurogenin 3 (Supplementary Figure S1b). All SYBR green primer sets were validated against human pancreatic RNA (Supplementary Figure S1c).

Glucose response of MafA-transfected cells

We then investigated whether regulation of the transcript levels in the MafA-transfected cells would be sensitive to glucose treatment. D12 MafA-transfected cells were treated with 16 mmol/l glucose for 3 hours. Total RNA was then

extracted for quantitative RT-PCR where we observed positive regulation in mRNA levels of the relevant transcription factors when compared with cells without glucose treatment (Figure 3a). Scatter plot fluorescence-activated cell sorting analysis (Figure 3b) demonstrated a significant increase in staining for insulin and C-peptide in MafA-transfected cells treated with glucose. Fluorescence-activated cell sorting analysis from a total of five independent experiments demonstrated a significant increase in expression of insulin and

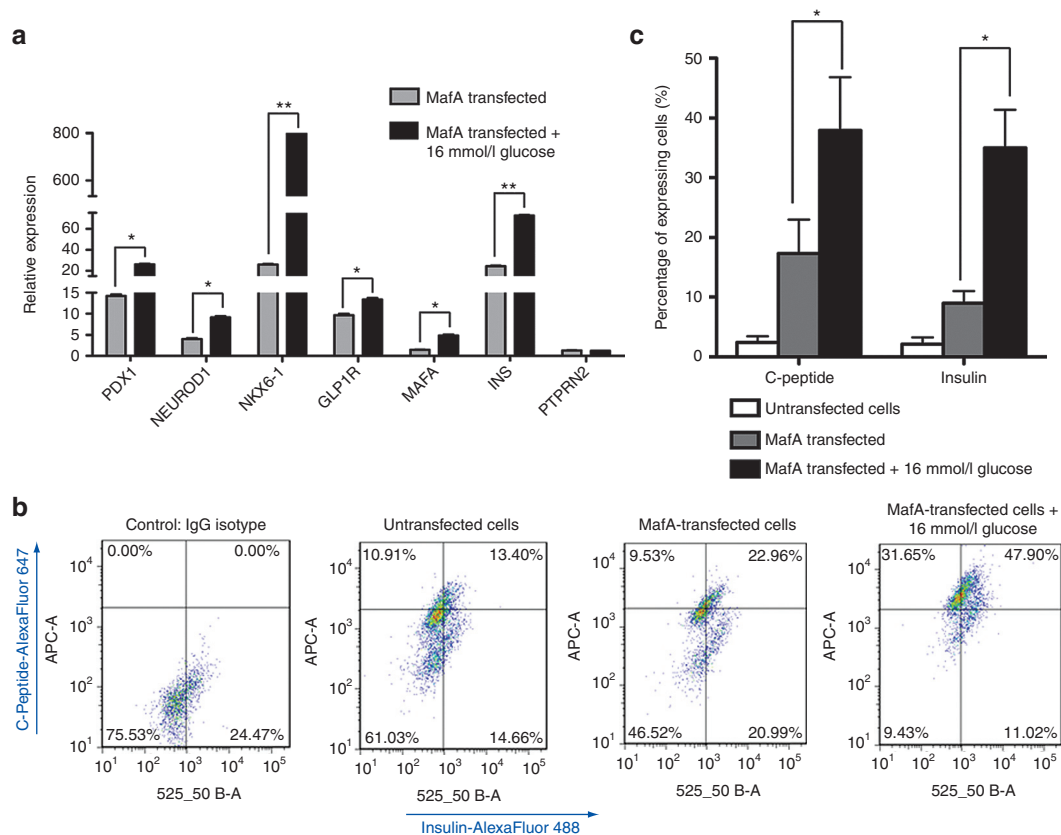


Figure 3 Glucose response in MafA-transfected cells. (a) MafA-transfected cells were exposed to 16 mmol/l of glucose for 3 hours and analyzed for transcripts determined to be necessary for insulin secretion. Expression levels of *PDX1*, *NEUROD1*, *NKX6-1*, *GLP1R*, *MafA*, *INS*, and *PTPRN2* increased up to 1.5-fold or more. Values represent the mean \pm SEM of three independent experiments in triplicate. * $P < 0.005$; ** $P < 0.0001$, paired Student's *t*-test, two-tailed at 95% CI. (b) Representative scatter plot of individual FACS-analyzed cells probed with insulin and C-peptide relative to control isotype IgG. (c) FACS-derived expression ratio from five independent differentiation experiments suggests that although there is no significant difference in expression between insulin or C-peptide, levels of both significantly increase (in excess of 35%) when MafA-transfected cells are exposed to glucose. Results are mean and SEM of five independent differentiation experiments. Mann–Whitney test was performed at 95% confidence. FACS, fluorescence-activated cell sorting; *GLP1R*, glucagon-like peptide 1 receptor; *INS*, Insulin; *MafA*, v-maf musculoaponeurotic fibrosarcoma oncogene homologue; *NEUROD1*, NeuroD; *NEUROG3*, Neurogenin 3; *PDX1*, Pancreatic and Duodenal homeobox 1; *PTPRN2*, Phogrin.

C-peptide in MafA-transfected cells by over 12% relative to untransfected cells. The percentage of positive expressing cells increased to over 30% in glucose-treated MafA-transfected cells relative to untreated MafA-transfected cells (Figure 3c). Individual flow cytometry data for untransfected CD34⁺ cells grown in the differentiation media is also shown for insulin and C-peptide (Supplementary Figure S2a,b, respectively).

Insulin and C-peptide release from MafA-differentiated cells

Having confirmed that MafA-transfected cells contained increasing levels of factors for insulin secretion by D12, we next determined whether they could respond biologically to increasing concentrations of glucose. An enzyme-linked immunoabsorbent assay (ELISA) specific for human insulin and C-peptide was performed. D12 MafA-transfected cells alongside D12-undifferentiated cells (control) were exposed initially to a low concentration of glucose (2.8 mmol/l) for 2 hours followed by exposure to a high concentration of glucose (16 mmol/l) for 3 hours (Figure 4a). The culture media

was then isolated for the ELISA onto 96-well plates. The data suggest that MafA-transfected cells secreted substantially more insulin (up to 18 pM/L) and C-peptide (up to 35 ng/ml) when exposed to high concentrations of glucose (Figure 4a). Total protein extracts from these cells were then separated by SDS-PAGE for a western blot to confirm that biologically active insulin (with both α and β chains) was expressed (Figure 4b). Immunofluorescent staining using antibodies specific for human insulin and C-peptide demonstrated a positive staining when MafA-transfected cells were stimulated with glucose (Figure 4c; Supplementary Figure S3a). We also observed accumulation of granular secretory like granules in the cytoplasm of MafA-transfected cells (Supplementary Figure S3b).

Taken together, we demonstrate here that a GMP compliant differentiation protocol using saRNAs in a serum-free environment enriched with growth factors and cytokines traditionally used for differentiation of insulin-secreting cells is sufficient to differentiate adult CD34⁺ cells towards one that expresses transcriptional factors specific for the endodermal pancreatic lineage.

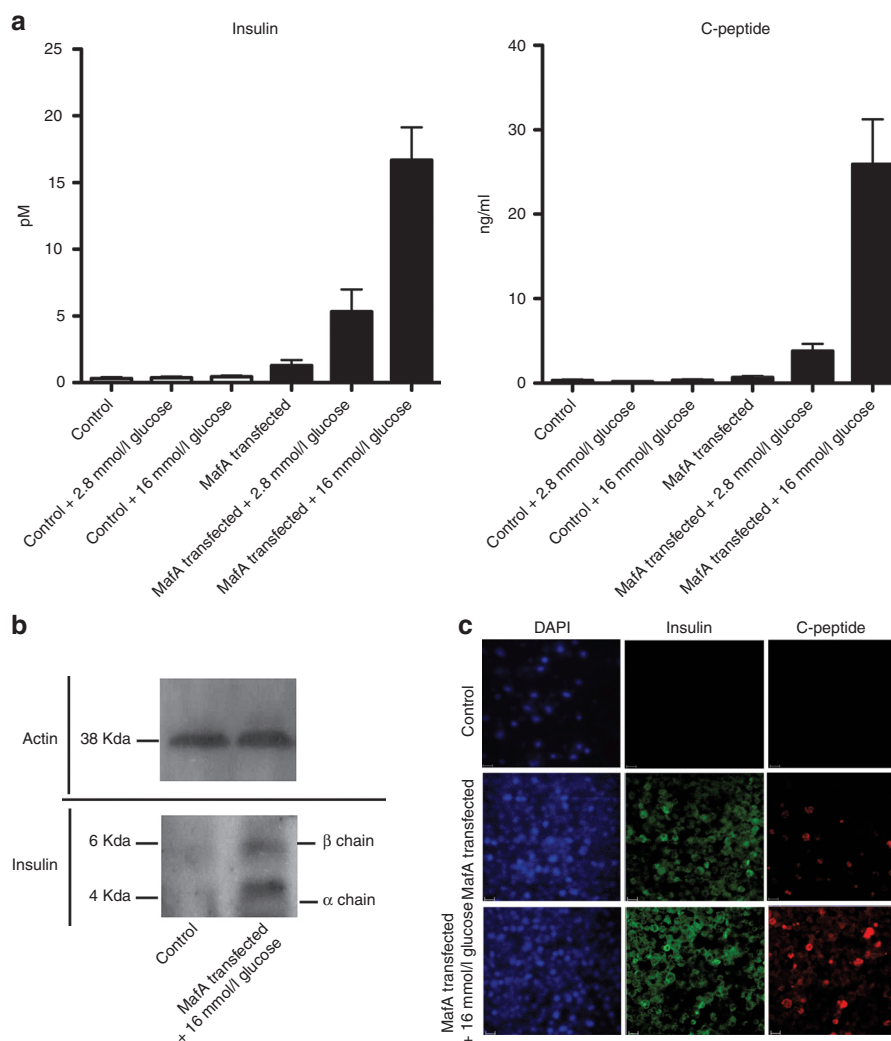


Figure 4 Insulin and C-peptide release from MafA-transfected cells. (a) ELISA demonstrating MafA-transfected cells respond to glucose stimulation when compared with undifferentiated CD34⁺ cells (control). The culture media from control and from day 12 MafA-transfected cells were recovered following exposure of cells to low glucose (2.8 mmol/l) followed by high glucose (16 mmol/l) pulse. The media was then transferred onto immunoabsorbent ELISA plates specific for human insulin and human C-peptide. The data suggest that MafA-transfected cells are able to respond to a glucose gradient by secreting insulin. (b) Day 12 MafA-transfected cells that were treated with 16 mmol/l glucose from as mention in a were isolated for total protein extraction and SDS-PAGE separation. A western blot probed with an antibody specific to human insulin demonstrates the presence of biologically active insulin with its α and β chain. (c) Confocal microscopy of undifferentiated (control) and MafA-transfected cells with or without glucose treatment. Both control and MafA-transfected cells (with or without glucose treatment) were then costained with anti-insulin and anti C-peptide.

Discussion

The primary therapeutic goal of preventing complications associated with diabetes is to maintain a stable glycemic content by reconstitution of endogenous insulin secretion. Islet transplantation therefore presents a feasible therapeutic alternative for metabolically labile patients with T1DM; however, shortage of organ donors does not make this a practical option. Many attempts have been made to develop surrogate insulin-secreting cells using embryonic stem cells, organ derived stem cells or inducible pluripotent stem cells.

The basic methodology of pushing stem cells towards the pancreatic endodermal lineage has previously been described by other groups. The addition of activin A, exendin-4, noggin, EGF, and bFGF during stages of the

differentiation process is known to drive stem cells towards an insulin-expressing phenotype.^{12,18–20} The current challenge however is the ability to maintain this expression to completion and generate cells that will respond to glucose using a GMP compliant serum-free method. This has not yet been achieved. We therefore demonstrate that a combination of GMP compliant growth factors and saRNA molecules can be used to transform human adult CD34⁺ cells into glucose sensitive and insulin-secreting cells. This novel use of saRNA oligonucleotides positively increases the transcript levels of MafA. Being the master switch for insulin expression in β -cells, MafA then induces expression of key endodermal endocrine factors including PDX1, Neurogenin 3 and NeuroD, MafA, glucokinase, glucagon-like peptide 1 receptor, SUR1-KATP, and phogrin. These factors are essential in supporting

glucose sensitivity and processing of insulin. This observation ties in with other reports by Wang *et al.* and Aramata *et al.* suggesting that the master regulatory potential of MafA can feasibly mediate transdifferentiation of progenitor cells towards an insulin secretory lineage.^{48,49} Moreover, our differentiation protocol also suggests a potential to upregulate sensitivity to the incretin hormone, GLP1 which is important in promoting pancreatic endocrine cell growth, in stimulating insulin release in the post-prandial state and in expansion of β -cell mass via islet cell neogenesis.⁴⁴ Therefore, our approach may be a possible avenue towards restoration of glucose sensitivity for type-2 diabetes and one that will be further investigated.

At the current stage of this promising approach to generating glucose responsive cells from hematopoietic derived CD34⁺ cells, our procedure demonstrates that up to 35% of MafA-transfected cells responds to glucose and achieved an insulin secretion content of approximately 18 pM/L by day 12. The next challenge will be to meet the normal adult insulin requirement of 57–79 pM/L (between meals) and to establish how these cells behave functionally *in vivo* and whether they may require encapsulation strategies or micro chamber technology to enhance cell survival beyond 12 days following transplantation.^{50,51} Despite the numerous hurdles ahead, the work performed here represents a significant step forward towards developing a potentially useful clinical tool for reconstituting endogenous insulin secretion with reduced possibility of rejection. Although this strategy may not completely remove the requirement for pharmacological insulin replacement formulations, it may provide a baseline to reduce the risks associated with severe diabetes.

Materials and methods

CD34⁺ stem cell source. Granulocyte colony-stimulating factor–mobilized peripheral blood cells were obtained from leukaphereses processed by the Stem Cell Laboratory, Hammersmith Hospital. A total of five healthy donor patients undergoing mobilization for transplantation in allogeneic recipients were used where cells were in excess of clinical requirements. Blood samples were obtained with informed patient consent and approved by the Hammersmith Hospital Research Ethics Committee. We found that the response of the cells used in the differentiation procedure was similar for all the five healthy donors.

CD34⁺ stem cell isolation. Human-mobilized peripheral blood samples were diluted in a ratio of 1:4 in Hanks' buffered saline solution (Gibco, Paisley, UK), the mononuclear cells were separated by centrifugation over a Lymphoprep (Axis-Shield, Dundee, UK) density gradient at 1,800 rpm for 30 minutes. The mononuclear cell fraction at the interface was aspirated and washed twice with HBSS, and finally with MACS buffer (Dulbecco's phosphate-buffered saline solution (DPBS) (Gibco) at pH 7.2 supplemented with 0.5% human serum albumin and 2 mmol/l EDTA). CD34⁺ cells were isolated using a CD34⁺ isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Adherent CD34⁺ progenitor cells were isolated as previously described with modification.^{38,39} Briefly, isolated CD34⁺ cells were added to 24-well

tissue culture treated dishes (Nunc, Loughborough, UK) at a density of $2.5\text{--}5 \times 10^5$ cells in α -MEM medium to isolate the adherent CD34⁺ cell population. After 30 minutes incubation, non-adherent cells were removed and adherent cells were rinsed three times with PBS. The adherent CD34⁺ cell population was grown in a serum-free medium-CellGro (CellGenix, Freiburg, UK) containing three cytokines: 250 ng/ml of stem cell factor, 250 ng/ml of interleukin-6, and 250 ng/ml of interleukin-3 (Invitrogen, Carlsbad, CA or CellGenix) in 0.5% penicillin/streptomycin antibiotics. Cells were incubated at 37 °C in humidified 5% CO₂ air.

Design of short-activating RNA oligonucleotides. The gene sequence of MafA was selected for designing short-activating RNA molecules for its specific activation. Four parameters were used: (i) targeting gene annotations from UCSC RefSeq database; (ii) targeted sequence from antisense RNA; (iii) promoter selection of antisense sequences; and (iv) identification of candidate short activating RNAs. First, the method downloads information about the target's genomic location, orientation, and transcriptional structure from available databases (RefSeq at UCSC). Second, given a database of RNA transcripts with known read direction, such as the UCSC Spliced EST track, our method searches the database for transcripts that are antisense to and in the vicinity of the target gene. More specifically, the method identifies antisense transcripts that (a) overlap the target's promoter and the target mRNA's 5' end; (b) overlap the target mRNA; (c) are at most 20-100 kb upstream of the target's transcription start site; or (d) are at most 20-100 kb downstream of the target's poly-adenylation site. The method uses these four criteria as hierarchical filters such that if it finds antisense transcripts that for example satisfy criterion (a), the method does not consider the three other criteria. Third, based on the target's transcription start site, the method downloads the antisense genomic sequence from a fixed size region upstream and downstream of the transcription start site. The typical region size used by the method is 500 nts upstream and downstream of transcription start site, but larger or smaller sizes can also be used. Fourth, the method designs siRNAs that give effective and specific downregulation of the antisense target sequence. The method (a) uses a siRNA design algorithm, such as GP boost,⁵² to identify candidate effective siRNAs; (b) removes all candidate siRNAs with aaaa, cccc, gggg, or uuuu motifs and GC content <20% or >55%; (c) removes all candidates that have Hamming distance less than two to all potential off-target transcripts; and (d) returns a given number of remaining non-overlapping siRNAs sorted by their predicted siRNA knockdown efficacy. The method returns the two highest scoring short activating RNAs for a given antisense target sequence. Paired short activating RNA oligonucleotides were annealed using 50 mmol/l Tris-HCl, pH 8.0, 100 mmol/l NaCl and 5 mmol/l EDTA following a denaturation step at 90 °C followed by a gradual anneal step to room temperature.

Differentiation procedure. A flow diagram of the differentiation procedure is shown in (Figure 1a). The commencement of the differentiation protocol involved supplementing the adherent subpopulation of hematopoietic adult CD34⁺ cells with

10 ng/ml of activin A (Sigma, Poole, UK) and 2.5 mmol/l glucose (Sigma, St Louis, MO) into serum-free CellGro media (Cell-Genix) containing Stem Cell Factor (Invitrogen), 250 ng/ml of interleukin-3 and interleukin-6 (Invitrogen) in 0.5% penicillin/streptomycin antibiotics for 72 hours at day 0. This was then followed by the addition of 11 mmol/l glucose, 5 ng exendin-4 (Sigma, St Louis, MO), 50 ng noggin (Sigma, St Louis, MO), 5 ng bFGF (Sigma, St Louis, MO), and 5 ng EGF (Sigma, St Louis, MO) at 72 hour intervals together with transfection 25 mmol/l of annealed short activating RNA oligonucleotide targeted to MafA (OmniCytte, London, UK) using Nanofectamine (PAA, Somerset, UK) following the manufacturer's instructions. This process was repeated three times at days 2, 5, and 8.

Isolation of total RNA. Total RNA extraction was carried out on non-adherent differentiated cells using the RNeasy-Micro kit (Ambion, Paisley, UK) following the manufacturer's instructions. Briefly, the cells were gently centrifuged followed by three pulses of sonication at Output 3 in Lysis buffer (Ambion). The cell lysates were then processed through an RNA binding column, followed by multiple washes and elution. The total RNA isolated was quantified by a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE).

For semi-quantitative RT-PCR. Total RNA of 500 ng was reversed transcribed using One Step RT-PCR (Qiagen, Hilden, Germany) following the manufacturer's instructions. Expression for PDX1, RFX6, MafA, insulin, and for loading control, the house keeping gene actin was performed by PCR using their respective primer pairs: PDX1- F: CGG AAG AAA AAG AGC CAT TG; R: GCC AGA GGA AGA GGA GGA CT following 59 cycles at 95 °C, 20 seconds; 60 °C, 20 seconds; 72 °C, 40 seconds. Rfx6- F: AAT GGC AGG GAG GCC CCC AA; R: CTG GGC TTG CAG GGA TGG GC following 39 cycles at 95 °C, 30 seconds; 55 °C, 30 seconds; 65 °C, 45 seconds. MafA-F: AGC TGG TGT CCA TGT CGG TGC G; R: TGG CAC TTC TCG CTC TCC AGA ATG T following 39 cycles at 95 °C, 45 seconds; 67.4 °C, 45 seconds; 72 °C, 45 seconds. Insulin (nested cycle, previously described)⁴²-F: GAC CTA CTG CCA CAA CAG CTA; R: GTT GCA GTA GTT CTC CAG CTG; following 40 cycles at 95 °C, 1 minute; 55 °C, 1 minute; 70 °C, 1 minute. For the nested step using F(nested): CTG TCC TTC TGC CAT GG; following 35 cycles at 50 °C, 1 minute; 95 °C, 1 minute; 58 °C, 45 seconds; 68 °C, 45 seconds in at 35 cycles. Actin-F: GAG AAA ATC TGG CAC CAC ACC; R: ATA CCC CTC GTA GAT GGG CAC following 37 cycles at 95 °C, 5 minutes; 60 °C, 30 seconds; 70 °C, 45 seconds; 72 °C, 10 minutes at 37 cycles.

For quantitative RT-PCR. Total extracted RNA of 500 ng was processed for elimination of genomic DNA followed by reverse transcription using the QuantiTect Reverse Transcription kit from Qiagen. The first strand cDNA synthesis was then amplified for quantitative analysis of PDX1 (NM_000209), Neurogenin 3 (NM_020999), NeuroD (NM_002500), NKX6-1 (NM_006168), MafA (NM_201589), glucokinase (NM_000162, NM_033507, NM_033508), GLP1R (NM_002062), SUR1, K-ATP component, *ABCC8* (NM_000352), phogrin (protein tyrosine phosphatase receptor (N-terminal polypeptide 2),

PTPRN2 (NM_002847, NM_130842, NM_130843), pro-Insulin (NM_000207, NM_001185097, NM_001185098), and the reference gene, human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (NM_002046) using QuantiFast SYBRGreen PCR Kit from Qiagen. Amplification was performed using Applied Biosystems 7900HT FAST-Real-Time System (Applied Biosystems, Paisley, UK) with 40 cycle conditions at 95 °C, 15 seconds and 60 °C, 45 seconds with a total volume of 25 µl per sample. Amplified products were then analyzed using Applied Biosystems RQ Manager 1.2.1. Four independent experiments were amplified in triplicates for quantitative analysis. Student's *t*-test scoring was performed at 99% confidence intervals.

Confocal microscopy. CD34⁺ cells were fixed for 20 minutes with 4% paraformaldehyde onto 1 cm glass cover slips followed by permeabilization with 0.2% TritonX100 for 15 minutes. The coverslips were washed three times with PBS followed by blocking with 5% mouse or rabbit serum for 45 minutes. Mouse raised anti-human insulin (1:200) (Sigma, Poole, UK) or rabbit raised anti-human C-peptide (1:200) (Abcam, Cambridge, UK), was added to the cells in 5% serum for 1 hour. Cells were washed three times in PBS followed by the addition of 5% serum for a further 15 minutes before incubation with the appropriate Alexa-488 conjugated or Alexa-594 secondary antibody (1:600) (Cell Signalling Technology, Hertfordshire, UK) for 1 hour. After five washes in PBS, coverslips were mounted onto glass slides with Vectashield containing 4',6'-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Slides were visualized on a Leica DM4000 at 60× magnification or Olympus epifluorescent CKX41 microscope. An average of five images was captured and compared with staining with the secondary conjugated antibody alone to confirm no presence of autofluorescence.

Insulin and C-peptide ELISA. MafA-transfected cells growing in suspension to day 12 were transferred to a preconditioning α -MEM media supplemented with 10 mmol/l nicotinamide, 50 ng IGF-II, 10 ng HGF, 25 ng exendin-4, and 10 ng activin A. Cells were incubated for 16 hours at 37 °C and 5% CO₂ before glucose stimulation. Glucose of 2.8 mmol/l was added for 3 hours followed by 16 mmol/l of glucose for a further 3 hours. The media was collected for total human insulin or human C-peptide ELISA (Millipore, Billerica, MA) following the manufacturer's instructions. Positive control used was as supplied by the kit.

Fluorescence-activated cell sorting analysis. Approximately 3 × 10⁶ of day 12 differentiated CD34⁺ cells were fixed and permeabilized using Fix&Perm fixation medium (Caltag Laboratories, Burlingame, CA) following the manufacturer's protocol. Briefly, after washing the cells in PBS, 100 µl of reagent A (fixation medium) were added and the cells were incubated for 15 minutes at room temperature. At the end of the incubation, the cells were washed in PBS then 100 µl of reagent B (permeabilization medium) were added for 15 minutes at room temperature. The cells were then resuspended in blocking buffer comprising PBS, with 5% fetal calf serum and 3% bovine serum albumin and incubated for 15 minutes. Cells were then incubated with antibodies against insulin (Abcam)

(1:350) and C-peptide (Santa Cruz Biotechnology, Dallas, TX) (1:350) for 1 hour. After a wash in 3% FBS/1% bovine serum albumin in PBS (wash solution), cells were incubated with 1 µg/ml goat anti-mouse Alexa Fluor-488 (green), goat anti-rabbit Alexa Fluor-488 (green), and goat anti-rabbit Alexa Fluor-647 (red) (all Invitrogen) in blocking solution for 45 minutes in the dark, washed with PBS and resuspended in PBS for flow cytometric analysis. Stained cells were analyzed using the BD LSRII flow cytometer with 525_50 B-A and APC lasers to view the Alexa Fluors-488 and 647 respectively. At least 10,000 cells were collected for each test sample to ensure a sufficient number of positive stained cells. FlowJo 7.6 software (FlowJo, Ashland, OR) was used for the analysis of the results (**Supplementary Materials and Methods**).

Supplementary material

Figure S1. (a) An insulin and C-peptide ELISA assay of MafA-transfected cells.

Figure S2. Individual and merged (in color) histograms of fluorescence-activated cell sorting analyzed cells probed with (a) anti-insulin or (b) anti-C-peptide.

Figure S3. A high magnification immunostaining of insulin and C-peptide in MafA-transfected cells with glucose treatment and control cells (undifferentiated CD34⁺ cells).

Materials and Methods

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